Current Biology 22, 707–711, April 24, 2012 ©2012 Elsevier Ltd All rights reserved DOI 10.1016/j.cub.2012.02.063

Report

Swarming Generates Rebel Workers in Honeybees

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Summary

According to kin selection theory, the colony kin structure of eusocial insects motivates workers' altruistic behaviors and therefore their sterility or restricted reproduction [1]. Indeed, theory and cross-species comparison confirm that workers engage in their own reproduction depending on relatedness among colony members [2, 3]. We show that in a honeybee colony, the workers switch from their typical altruistic role to a more selfish one if at their larval stage there are environmental cues of an upcoming decline in intracolony relatedness. This happens inevitably when a colony multiplies by swarming and replaces the mother queen with her daughter, because the mother queen's workers are faced with rearing the sister queen's offspring related to them half as much as between sisters. Workers developing from the mother queen's eggs immediately after swarming, in a temporarily queenless colony, had more ovarioles in their ovaries and less-developed hypopharyngeal glands producing brood food than control workers reared in queenright conditions. These "rebel" workers were more engaged in laying their own male-determined eggs than in rearing offspring, whether or not the sister queen was present in the colony. The finding of this previously unknown rebel strategy confirms that kin selection shapes both cooperation and conflict in honeybee societies.

Results and Discussion

The honeybee (*Apis mellifera*), an insect whose biology is very well known, draws widespread interest not only as a honey producer and the main pollinator of crops but also as a model organism for testing evolutionary theories and hypotheses [4–7]. In all of these models, reproductive altruism via sterility or restricted reproduction of workers is posited as the defining character of bee societies, and kin selection theory is normally used to explain altruistic strategies of colony members [8, 9]. Because in hymenopteran insects, including those that form societies (ants, bees, wasps), the males develop from unfertilized eggs, a diploid female is less related to her daughters and sons (0.5) than to full sisters (0.75). These phenomena can generate cooperation [1, 10] but also conflicts, as may be the case between workers of a polyandrous queen honeybee. Workers more related to brothers (i.e., mother queen's sons; 0.25 relatedness) than to other workers' sons (i.e., nephews; 0.14 relatedness, half of the 0.28 relatedness among workers if the queen has an average of 17 mates [11]) police one another against producing male-determined eggs [12, 13]. Consequently, the presence of a reproducing queen along with worker policing successfully suppresses workers' reproduction, because only 0.01%–0.1% of workers in a well-functioning colony have active ovaries [14]. The situation changes if a queen dies and her colony has no chance to rear a new queen, because then 5%–24% of the workers begin laying unfertilized male-determined eggs [15].

The tendency toward an increase in the number of workers with active ovaries seems not to be stable across the season [16, 17]. There are suggestions that their number increases in early summer after swarming [18], which is the only natural means of colony multiplication and which is initiated when workers begin rearing several young queens (workers' sisters). Before the first new queen emerges, the old mother queen leaves her native nest together with part of the adult workers [19]. The abandoned workers care for the pupae, larvae, and eggs of younger worker cohorts and the developing sister queens. Because cues about the queen's presence are exchanged during food distribution among colony members [20], the absence of an adult queen is quickly perceived by workers [21, 22] and likely also by larvae [23, 24]. In the few days following swarming, the accepted young queen kills other newly hatched and unhatched sister queens, performs the nuptial flight during which she mates with unrelated drones, and then begins laying eggs nursed by sister and half-sister workers. This situation generates conflict, because the relatedness of older-generation workers to the new sister queen's offspring is reduced by half. The mother queen used to produce sisters and brothers, whereas a sister queen produces nieces and nephews.

The kin selection theory predicts that relatedness is crucial in determining the life-history strategies of colony members [1, 25] and that a decrease in relatedness generates self-interested behaviors and conflicts [3]. If this is so, a mother queen's daughters should adopt a different strategy following a queen replacement at swarming. They should develop the ability to lay male-determined eggs regardless of the apparent conflict with the elected sister queen and her future offspring. These "rebel" workers could also pass on their own genes more effectively than other workers do when a young queen dies, as occurs during 14%–35% of mating flights [26, 27]. The expression of such selfish traits would need to be induced early in larval development before the standard worker-caste phenotype is fixed. That such a strategy really exists seems to be supported by data from only eight larvae, which suggested that workers reared in an artificially orphaned honeybee colony developed more ovarioles than larvae reared with a queen [28].

We tested these predictions by observing workers in two independent experiments: experiment 1 with naturally swarming honeybee colonies, and experiment 2 with artificially split colonies that mimicked temporary lack of a queen at swarming. The experiments were of similar design. First, larvae destined to be workers were developed in a colony either with or without a mother queen, to compare the reproductive potential and the brood care readiness of newly emerged adults. Next, these workers were placed in either a queenright or a queenless colony to compare their ovarian and hypopharyngeal gland development, which indicates their capacity for reproductive and brood care activity.

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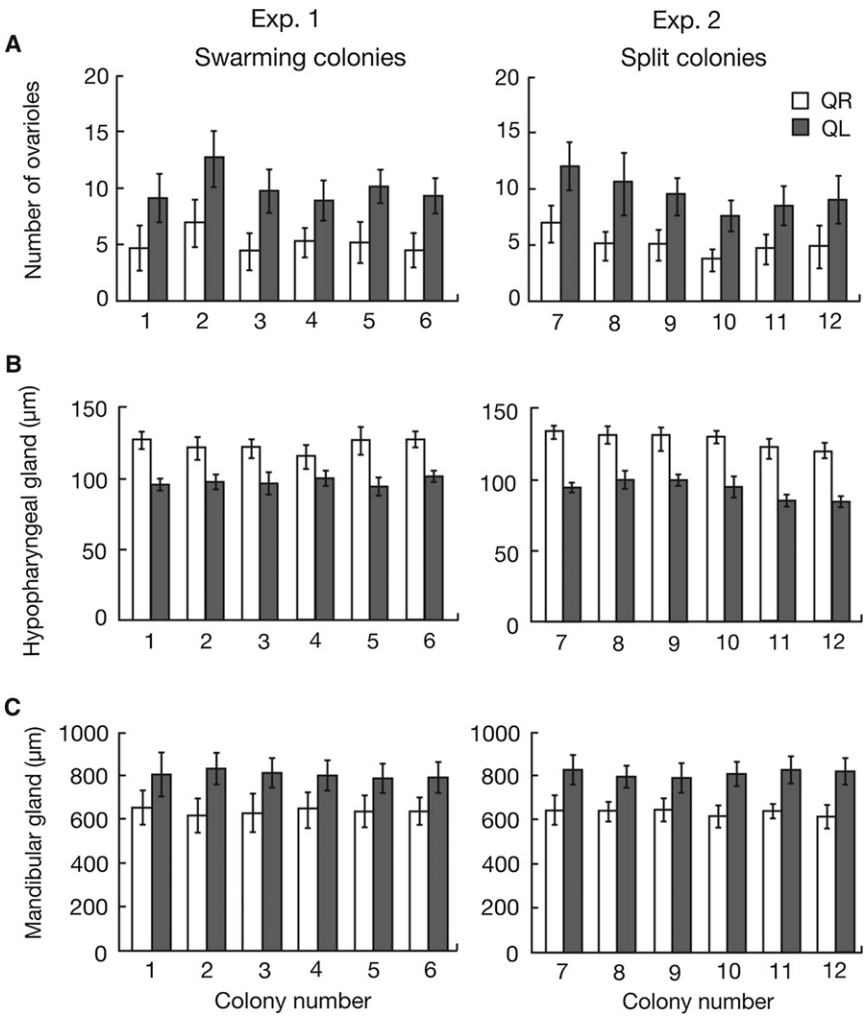

Figure 1. Newly Emerged Honeybee Workers Reared as Larvae in Queenright or Queenless Colonies

For details, see Experimental Procedures and Supplemental Experimental Procedures. The following abbreviations are used: QR, queenright; QL, queenless.

(A) Number of ovarioles (mean ± SD). Experiment 1, naturally swarming colonies (factorial ANOVA, $F_{1,5} = 341.1$; $p < 0.001$; n = 360; Table S1B); experiment 2, artificially split colonies (factorial ANOVA, $F_{1,5} = 261.4$; $p < 0.001$; n = 360; Table S1B).

(B) Size of hypopharyngeal glands (mean ± SD). Experiment 1, naturally swarming colonies (factorial ANOVA, $F_{1,5} = 113.2$; $p = 0.001$; n = 360; Table S1C); experiment 2, artificially split colonies (factorial ANOVA, $F_{1,5} = 1054.9$; $p < 0.001$; n = 360; Table S1C).

(C) Size of mandibular glands (mean ± SD). Experiment 1, naturally swarming colonies (factorial ANOVA, $F_{1,5} = 258.2$; $p < 0.001$; n = 360; Table S1D); experiment 2, artificially split colonies (factorial ANOVA, $F_{1,5} = 386.6$; $p < 0.001$; n = 360; Table S1D).

There are studies suggesting that larvae in orphaned colonies may receive more food [29], but testing larval development on naturally swarming (experiment 1) and artificially split (experiment 2) colonies, we found that workers reared as larvae with and without a queen did not differ with respect to body mass (mean ± SD; experiment 1: 116.6 ± 10.1 and 122.2 ± 7.3 mg reared with or without a queen, analysis of variance [ANOVA], $p = 0.072$, n = 1,952; experiment 2: 124.7 ± 9.1 and 122.8 ± 8.2 mg reared with or without a queen, ANOVA, $p = 0.291$, n = 2,163; see Table S1A available online). More important, however, was that comparing the same groups of workers with respect to the number of ovarioles in their ovaries, we found that workers reared without a queen had significantly more ovarioles (Figure 1A; Table S1B). Apparently the increase in the number of ovarioles in newly emerged workers reared as larvae without a queen was induced during the larval stage at the expense of other tissues. In support of this, we found that these workers developed smaller hypopharyngeal glands than did workers raised as larvae with a queen (Figure 1B; Table S1C), but we also noted that they enlarged their mandibular glands (Figure 1C; Table S1D), as queens do [19]. All of this made them more queen-like than normal workers, justifying the term "rebel." A similar example of biasing caste fate by reallocation of resources during larval development has been documented in some *Melipona* bees, where many larvae develop into queens instead of sterile workers against the interest of the colony as a whole [30].

The number of ovarioles is a good fertility predictor in solitary insects [31] and bees [4, 32]; in this study, we verified whether the higher number of ovarioles in workers reared as larvae without a queen indicated an elevated potential for egg laying. We compared the two groups of 15-day-old workers reared as larvae with or without a queen in both queenright and queenless conditions. As expected, in naturally swarming as well as in artificially split colonies where a queen was present, the frequency of workers with active ovaries was significantly higher in rebels (Figure 2; Tables S2A and S2B). A similar tendency (toward more developed ovaries in rebels) was observed among adult workers in queenless colonies from both experiments (Figure 2; Tables S2C and S2D). The appearance of workers with mature ovaries among nonrebels in orphaned colonies, albeit at low frequency, is not surprising. It is known that workers lay eggs if a colony loses its queen and there is no chance to rear a new queen [16, 22]. Reproduction by rebels in queenright colonies is much more striking, because it contradicts a common belief that the presence of a queen effectively inhibits worker oogenesis [16, 22]. Our experiment with artificially divided colonies made it clear that for workers, it was crucial whether during their larval feeding period they were in a colony with or without a queen, even if after this short time without a queen they returned to their native colony and hatched in the mother queen's presence (experiment 2). The finding of rebels laying eggs in the mother queen's presence is in line with suggestions that kin recognition does not play an important role in honeybee worker strategies [33, 34]. Workers have apparently been selected to recognize queen replacement, and the predictable reduction of their relatedness to the offspring of a new queen, and they

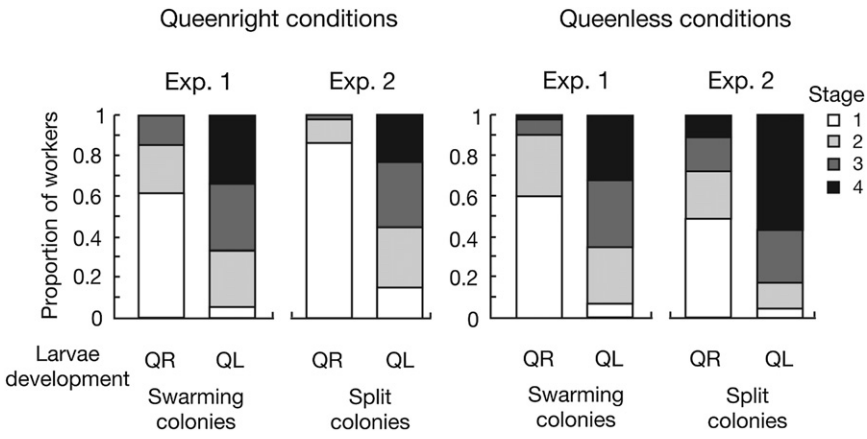

Figure 2. Ovary Activation in 15-Day-Old Workers Developed as Larvae in Queenright or Queenless Colonies and Then Developed as Adults in Queenright or Queenless Conditions

Each bar represents pooled data for three colonies. Stages of ovary activation are marked with different shading and numbers from 1 (nonactive ovary) to 4 (mature ovary with at least one egg; for details, see Experimental Procedures and Supplemental Experimental Procedures). The following abbreviations are used: QR, queenright; QL, queenless. G test, differences between workers developed in QR and QL colonies: experiment 1, QR conditions, df = 3; $G \geq 17.3$; $p < 0.001$ in colonies tested independently (Table S2A); experiment 2, QR conditions, df = 3; $G \geq 32.9$; $p < 0.001$ in each of three tested colonies (Table S2B); experiment 1, QL conditions, df = 3; $G \geq 16.2$; $p \leq 0.001$ in each of three tested colonies (Table S2C); experiment 2, QL conditions, df = 3; $G \geq 30.6$; $p < 0.001$ in each of three tested colonies (Table S2D).

use the absence of queen pheromones as a proximate cue to achieve this.

In swarming colonies, there were some workers with slightly swollen ovaries among those that as larvae had been fed in the queen's presence and met a queen in adulthood (Figure 2), but this is easy to explain, because these 15-day-old workers spent most of their adult life (8 to 12 days, depending on the colony) in the colony before the new queen began to lay eggs. The rebels that emerged in the same swarming colonies 7 days later met a queen at a much younger age (1–5 days old); nevertheless, more of them possessed active ovaries. This confirms suggestions that before a young queen starts laying her eggs, the workers behave as if in an orphaned colony [24, 35]. The selfishness of adult rebels expressed in their reproductive activity is additionally supported by other factors. They had less developed hypopharyngeal glands (Figure 3A; Table S3A), which suggests low production of brood food [36] and restricted nurse activity [37], as well as larger mandibular glands (Figure 3B; Table S3B) producing queen-like pheromones [24]. These results, which were similar regardless of whether rebels met a queen in their adulthood or not, correspond with those of the first stage of both experiments (Figures 1B and 1C) and show that rebels differ in their physiology from other workers, probably because they follow a different developmental pathway. An earlier study suggested a positive rather than a negative relationship between ovary activation and hypopharyngeal gland development, explained by an elevated level of vitellogenin [38], a conserved yolk precursor synthesized by most laying insect females [39].

Based on our results, we offer a novel interpretation of the inclusive fitness consequences of swarming. Larvae hatching from eggs laid during the 3 days prior to swarming usually complete feeding in the absence of a queen [19] (3 days of egg incubation and 6 days of feeding larvae), and they definitely complete feeding before a new queen begins laying eggs. Even if, as much as a week prior to swarming, the mother queen receives less food and thus lays fewer eggs [19], hundreds of potential rebels will still mature following her departure. The absence of a queen during larval feeding triggers the rebels' strategy, whereas the dramatic drop in relatedness between old-generation workers and the new queen's offspring seems to be the ultimate factor justifying the shift in resource reallocation to reproductive tissue in rebels. Consequently, phenotypic plasticity emerges because, unlike other workers, the rebels are ready to improve their direct fitness (more ovarioles, bigger mandibular glands) at the expense of their contribution to indirect fitness (smaller hypopharyngeal glands). Their strategy is successful whether a new queen reigns over a colony or dies during the mating flight. The problem is that the rebels act in conflict not only with the sister queen but also with other workers originating from both a mother queen and in particular the sister queen. This is because each worker benefits more from her own reproduction than from supporting the reproduction of her rebel sister or rebel aunt. Because honeybee workers are short lived, the time window for expressing selfish worker traits lasts as long as rebels reproduce. Soon the new queen's offspring developing in the presence of their new mother queen will be focused on indirect fitness.

Our finding that intracolony relatedness drives the development of reproductive traits in workers contributes to the continuing debate about the role of kin selection in shaping worker strategies in eusocial insect colonies [9, 40]. We have revealed the effects of the short-lived conflict that emerges after swarming, between the old generation of workers and the descendants of their sister queen. This conflict and its effects, predicted by kin selection theory, have implications for the evolution of worker life-history strategies, and in particular for the regulation of their reproduction, a crucial point in almost all models with the honeybee as an example.

Experimental Procedures

We performed our experiments in May and June 2010 in the experimental apiary of the Institute of Environmental Sciences at the Jagiellonian University. In two experiments of similar design, we first compared newly emerged workers that developed as larvae with or without a queen. We then compared the ontogeny of these two groups of workers after 15 days of their adult life.

Experiment 1, Involving Six Naturally Swarming Colonies (Colonies 1–6)

After the colonies swarmed (day 0), we studied two groups of workers from each of the colonies: (1) those that emerged 12 days after swarming and fed as larvae in the presence of the mother queen, that is, before the mother queen left the native nest along with her swarm, and (2) those that emerged 19 days after swarming and fed as larvae in the absence of the queen, just after the mother queen left the native nest with the swarm and before a new young queen emerged. All of these newly emerged workers were weighed,

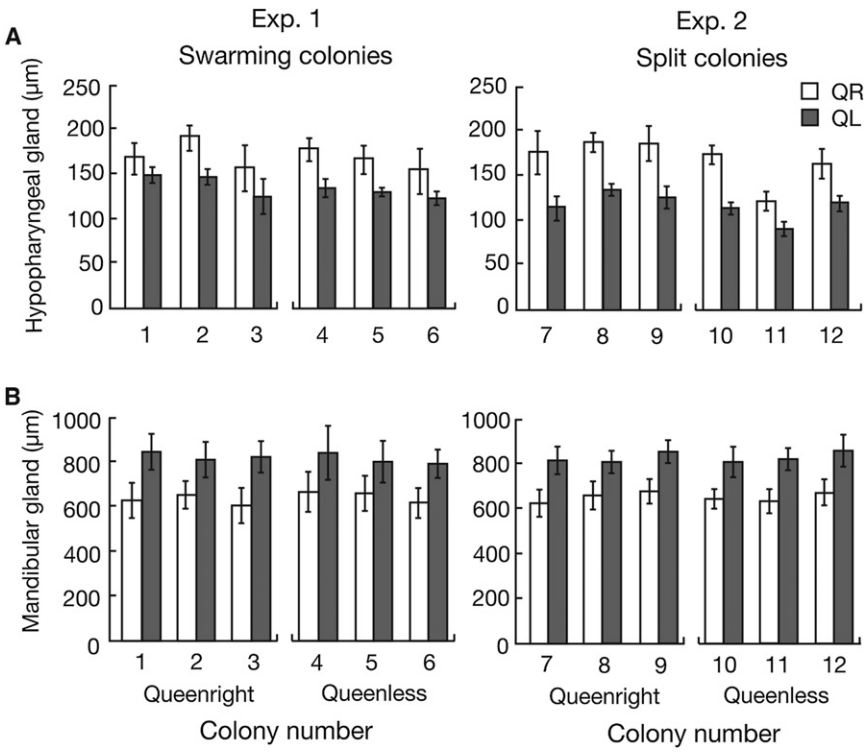

Figure 3. Fifteen-Day-Old Honeybee Workers Reared as Larvae in Queenright or Queenless Colonies and Then Developed as Adults in Queenright or Queenless Conditions

For details, see Experimental Procedures and Supplemental Experimental Procedures. The following abbreviations are used: QR, queenright; QL, queenless.

(A) Size of hypopharyngeal glands (mean ± SD). Factorial ANOVA, differences between workers developed in QR and QL colonies: experiment 1, QR conditions, $F_{1,2} = 21.6$; $p = 0.039$; $n = 172$; experiment 1, QL conditions, $F_{1,2} = 85.8$; $p = 0.011$; $n = 180$; experiment 2, QR conditions, $F_{1,2} = 418.9$; $p = 0.0024$; $n = 180$; experiment 2, QL conditions, $F_{1,2} = 24.5$; $p = 0.038$; $n = 180$ (Table S3A).

(B) Size of mandibular glands (mean ± SD). Factorial ANOVA, differences between workers developed in QR and QL colonies: experiment 1, QR conditions, $F_{1,2} = 107.2$; $p = 0.009$; $n = 172$; experiment 1, QL conditions, $F_{1,2} = 90.8$; $p = 0.011$; $n = 180$; experiment 2, QR conditions, $F_{1,2} = 162.4$; $p = 0.006$; $n = 180$; experiment 2, QL conditions, $F_{1,2} = 255.9$; $p = 0.004$; $n = 180$ (Table S3B).

and 30 bees from each group were dissected to evaluate whether larval development had produced a different kind of worker phenotype. In each worker, we counted the number of ovarioles in two ovaries and measured the size of the hypopharyngeal glands and the size of the mandibular glands. The remaining newly emerged bees were marked and returned to their native colonies to assess the effects of adult worker development. In three colonies (colonies 1, 2, and 3), the new young queens emerged after swarming, and after the mating flight they started laying their eggs (queenright conditions). The new eggs were observed at days 20, 21, and 24 in colonies 1, 2, and 3, respectively. In three other colonies (colonies 4, 5, and 6), the new young queens were removed from their queen cells just before they hatched (queenless conditions). When the tested workers were 15 days old, 30 of them (22 in colony 3, queenless at the larval stage) from each group and both queenright and queenless colonies were dissected.

Experiment 2, Involving Six Artificially Split Colonies (Colonies 7–12)

The queen of each colony was confined to two experimental frames to produce eggs of similar age. Three days later, the colony was divided into queenright and queenless subunits, each with one experimental frame (day 0). To ensure similar conditions for feeding the experimental larvae, the queen in the queenright subunit was again confined to two nonexperimental frames (day 0), changed after 3 days, and her newly laid eggs were removed. The queen was released on day 6. The subunits were reunited when the worker cells on the experimental frames were sealed (day 9). All newly emerged workers from two experimental frames were weighed (day 19), and 30 from each frame (two groups) were dissected to assess the effects of larval development as in experiment 1. The remaining workers were marked and returned to their native colonies, three of which (colonies 10, 11, and 12) were dequeened (day 19). When the workers were 15 days old, 30 of them from each group (different frames) were dissected to evaluate the effects of adult worker development as in experiment 1.

Sampled workers and data analysis are described in the Supplemental Experimental Procedures.

Supplemental Information

Supplemental Information includes three tables and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.cub.2012.02.063.

Acknowledgments

We thank Marcin Czarnoleski, Michael Jacobs, Elżbieta Krol, Jan Kozlowski, Adam Lomnicki, Hajnalka Szentgyörgyi, and two anonymous reviewers for suggestions and comments. This work was funded by the Jagiellonian University grant DS/BiNoZ/INoŚ/761/10-11 and by the European Commission under grant 244090-STEP-CP-FP (Status and Trends of European Pollinators; www.step-project.net) within the European Union Seventh Framework Programme.

Received: December 13, 2011
Revised: January 23, 2012
Accepted: February 21, 2012
Published online: March 29, 2012

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